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<b>(21) International Application Number:</b> PCT/US95/07784 <b>(22) International Filing Date:</b> 19 June 1995 (19.06.95)  <b>(30) Priority Data:</b> 264,861 23 June 1994 (23.06.94) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 264,861 (CON) Filed on 23 June 1994 (23.06.94)  <b>(71) Applicant (for all designated States except US):</b> MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MOTAMEDI, Haideh [IR/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). SHAFIEE, Ali [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(74) Common Representative:</b> MERCK & CO., INC.; Patent Dept., 126 East Lincoln Avenue, Rahway, NJ 07065 (US).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> EXPRESSION CASSETTES USEFUL IN CONSTRUCTION OF INTEGRATIVE AND REPLICATIVE EXPRESSION VECTORS FOR STREPTOMYCES		
<b>(57) Abstract</b> <p>The present invention is directed to expression cassettes useful in gene expression studies in both homologous and heterologous <i>Streptomyces</i> strains. More specifically the present invention is directed to the construction of two <i>Streptomyces lividans</i>-recombinant strains capable of producing 31-O-desmethylFK-506 O-methyltransferase, which methylates 31-O-desmethylFK-506 to FK-506. In addition, the present invention is directed to a process for the specific methylation of 31-desmethyl-FK506 to FK506.</p>		

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TITLE OF THE INVENTION

EXPRESSION CASSETTES USEFUL IN CONSTRUCTION OF  
INTEGRATIVE AND REPLICATIVE EXPRESSION VECTORS FOR  
STREPTOMYCES

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BACKGROUND OF THE INVENTION1. Field of the Invention

The present invention relates to the construction of  
expression cassettes for use in gene expression studies in both  
homologous and heterologous *Streptomyces* strain background.  
Specifically this invention describes construction of two *Streptomyces*  
*lividans*-recombinant strains capable of producing 31-O-desmethylFK-  
506 O:methyltransferase, which methylates 31-O-desmethylFK-506 to  
FK-506.

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2. Brief Description of Disclosures in the Art

The economic potential of DNA recombinant technology in  
producing unlimited amount of the pharmaceutically valuable peptides  
and proteins is vast. For example, the biologically active peptides and  
proteins such as insulin, erythropoietin, granulocyte-colony stimulating  
factor (G-CSF) and other important growth factors can now be  
produced in an unlimited quantities in bacteria, yeast and mammalian  
cell lines. Similarly, unlimited amounts of catalytic proteins (enzymes)  
may also be produced. This is significant because the use of enzymes as  
catalytic reagents in the synthesis of organic compounds are becoming  
increasingly practical and popular, not only because of economic  
factors, but also due to convenience in carrying out the chemical  
reactions, and environmental concerns where the use of toxic organic  
chemicals and solvents needs to be reduced or eliminated. In addition,  
enzymes are capable of catalyzing certain reactions that might otherwise  
be impossible or difficult to carry out by traditional organic reagents.  
To exploit DNA recombinant technology, however, it is essential that a  
large scale fermentation technology is available. Furthermore, it is  
equally critical that the bioconverting cultures and/or purified catalytic

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proteins capable of catalyzing particular organic reactions are accessible.

Regarding large scale fermentation, the technology is well developed for the microorganisms belonging to the genus *Streptomyces*; a large number of the secondary metabolites and extracellular enzymes are commercially produced by the fermentation of this microorganism. With regard to the availability of the bioconverting cultures and/or purified proteins, these cultures and proteins have to be discovered, isolated and/or made by using a genetic engineering approach. There is a long felt need in the art for different cultures which are capable of catalyzing a variety of organic reactions. The present invention meets this need by providing expression systems for the introduction of a single gene into the *Streptomyces* chromosome which enables the host organism to produce proteins with particular desirable catalytic properties. Furthermore, the present expression systems may be transferred as cassettes onto low as well as high copy *Streptomyces* replicating plasmids for the production of large quantities of a desired compound.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Restriction map of FKMT2 coding region and surrounding area of *Streptomyces* sp. MA6548 (ATCC No. 53770), wherein the arrow indicates location, relative size and direction of transcription of FKMT2 gene.

Figure 2: Schematic representation of the components used in the construction of the integrative expression vector, wherein:

"Perm E\*" is a promoter fragment derived from erythromycin resistance gene of *Saccharopolyspora erythrea* (Bibb, M.J., et al., Mol. Gen. Genetic 199: 26-36 (1985), Bibb, M.J., et al., Fifth Int'l Symp. on the Genetics of Industrial Microorganisms, 309-318 (1986));

"RBS" is a ribosomal binding site;

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"NdeI" is a restriction recognition site containing ATG translational start codon;

"MCS" represents multiple cloning sites;

5 "Gene X" represents the gene of interest cloned in the coding orientation relative to Perm E\* promoter as shown by the arrow;

"Ter" is a terminator derived from transcription termination region of FKMT2 gene of *Streptomyces* MA6548 (ATCC No. 53770);

10 "hyg" is the gene encoding hygromycin phosphotransferase derived from *S. hygroscopicus* (Malpartida, F., et al., Biochem. Biophys. Res. Commun., 117, 6-12 (1983));

"IE" is an integration element to direct insertion of the constructed vector into the chromosome of *Streptomyces*; and

15 "ori" is the origin of DNA replication, required for propagation of the integrative vector in *E. coli*.

Figure 3: Depiction of two constructs in which the RBS was within two different sequence contexts (a, b). The nucleotide sequence (SEQ ID NO:1) of the ribosome binding site (RBS) is

AGGAGGA

20 and it is used in the construction of expression cassettes in two (a, b) sequence contexts.

25 Figures 4a and 4b: Schematic representation of the construction of integrative expression vectors pHM6a and pHM6b that express the enzyme FKMT2.

30 Figure 5: Schematic representation of the construction of expression cassettes pHM7a and 7b and integrative expression cassettes pHM8a and pHM8b. Unique restriction sites are shown in bold letters.

#### SUMMARY OF THE INVENTION

The present invention is directed to expression cassettes which are useful as vehicles for the transfer of single copy of desired genes through their integration into the chromosome of *Streptomyces*.

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Furthermore, the instant expression vectors are useful for the transfer of multiple copies of a gene of interest, via a low copy or high copy replicative plasmid, into any *Streptomyces sp.* More specifically, the present invention is directed to the construction of two *S. lividans* recombinant strains, useful for the production of 31-desmethyl FK506-O-methyltransferase enzyme, which itself is useful for the conversion of 31-desmethyl-FK506 to FK506. In addition, the present invention is directed to a process for the specific methylation of 31-desmethyl-FK506 to FK506.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to integrative expression constructs for heterologous and homologous expression of genes in a *Streptomyces sp.* These expression constructs are useful for the expression in *Streptomyces* of a desired protein of either prokaryotic or eukaryotic origin.

An integrative expression cassettes for the expression of a gene of interest in *Streptomyces* comprises:

- (1) a strong promoter;
- (2) a synthetic ribosome binding site;
- (3) a translation start site embedded in *NdeI* recognition site;
- (4) a multiple cloning site; and
- (5) a transcription terminator downstream from the cloning site;

in association with:

- (i) an integration element to direct insertion of the construct into the *Streptomyces* genome;
- (ii) a drug marker for selection both in *E. coli* and *Streptomyces*; and
- (iii) an *E. coli* replicon;

wherein the components (1) - (5) are associated in the specified order and each of the components (i), (ii), and (iii) may be independently associated either upstream or downstream of components (1) - (5).

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The expression cassette more specifically comprises the following elements:

- (1) a strong constitutive promoter *PermE\**;
- 5 (2) a synthetic ribosome binding site derived from the RBS region of the gene encoding elongation factor Tu (*tufI*) of *Streptomyces ramocissimus*;
- (3) an ATG start codon contained within the *NdeI* cloning site;
- (4) a multiple cloning site, which gives additional cloning sites into which a gene of interest may be cloned;
- 10 (5) a transcription terminator derived from the terminator region of a methyltransferase gene involved in the biosynthesis of FK-506;

in association with:

- 15 (i) a hygromycin resistance gene which acts as selectable marker by inactivating the drug hygromycin B;
- (ii) a minicircle as an integration element; and
- (iii) a Col E1 replicon;

20 wherein the components (1) - (5) are associated in the specified order and each of the components (i), (ii), and (iii) may be independently associated either upstream or downstream of components (1) - (5).

In an alternative embodiment of the present invention, the expression cassette more specifically comprises the following elements:

- 25 (1) a strong constitutive promoter *PermE\**;
- (2) a synthetic ribosome binding site derived from a consensus *E. coli*-RBS sequence;
- (3) an ATG start codon contained within the *NdeI* cloning site;
- 30 (4) a multiple cloning site, which gives additional cloning sites into which a gene of interest may be cloned;
- (5) a transcription terminator derived from the terminator region of a methyltransferase gene involved in the biosynthesis of FK-506;

in association with:

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- (i) a hygromycin resistance gene which acts as selectable marker by inactivating the drug hygromycin B;
- (ii) a minicircle as an integration element; and
- (iii) a Col E1 replicon;

wherein the components (1) - (5) are associated in the specified order and each of the components (i), (ii), and (iii) may be independently associated either upstream or downstream of components (1) - (5).

In the instant expression cassettes, the strong constitutive promoter PermE\* is a promoter-up mutant (see Bibb, M.J., Janssen, G.R., "Unusual features of transcriptions and translation of antibiotic resistance genes in antibiotic producing *Streptomyces*", In Fifth International Symposium on the Genetics of Industrial Microorganisms, 309-318 (1986)) derived from the wild type promoter for erythromycin resistance gene of *Saccharopolyspora erythraea* (see Bibb, M.J., Ward, J.M., Cohen, S.N., "Nucleotide sequence encoding and promoting expression of 3 antibiotic resistance genes indigenous to *Streptomyces*", Mol. Gen. Genetic 199: 26-36 (1985)).

The synthetic ribosome binding site is derived from the RBS region of the gene encoding elongation factor Tu (*tufI*) (a highly expressed gene in *Streptomyces*) (Vijgenboon, E., et al., Microbiology, 140, 983-998 (1994)) and/or a consensus *E. coli*-RBS sequence (Gold, L. and Stormo, G.D., Methods in Enzymology, 185, 89-93 (1990)) to ensure efficient translation.

The minicircle is a *Streptomyces* integration element derived from *Streptomyces coelicolor* and it functions to direct the insertion of the vector into the *Streptomyces* genome. This integration element is known to mediate integration of foreign genes in many *Streptomyces* that apparently have the attachment site for minicircle.

The Col E1 replicon is present for propagation in *E. coli*.



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A specific embodiment of the present invention is directed to an expression vector for the overexpression of a gene of interest in *Streptomyces* comprising:

- 5 (1) a strong constitutive promoter, Perm E\*, derived from erythromycin resistance gene of *Saccharopolyspora erythrea*;
  - (2) a synthetic ribosomal binding site (RBS) which include the heptamer (SEQ ID NO:2)  
AGGAGGA  
10 in particular, wherein the synthetic ribosomal binding site (RBS) is present in the sequence context as depicted in sequence context (a) of Figure 3, or in the sequence context as depicted in sequence context (b) of Figure 3;
  - 15 (3) a translation start site/cloning site which is a *NdeI* restriction site bearing a (SEQ ID NO:3)  
CATATG  
recognition site and that serves both as cloning site and translation start site;
  - 20 (4) a multiple cloning site; and
  - (5) a terminator derived from the terminator region of the gene encoding FK506 methyltransferase, FKMT2 (isolated from MA6548) that is located downstream from the cloning site to prevent readthrough transcription;
- in association with:
- 25 (i) a drug marker, which is hygromycin phosphotransferase and which is expressed both in *E. coli* and *Streptomyces* and confers hygromycin resistance to the host by inactivation of hygromycin B;
- 30 wherein the components (1) - (5) are associated in the specified order and the component (i) may be associated either upstream or downstream of components (1) - (5).

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The desired DNA may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, as well as translational elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant protein. Techniques for such manipulations can be found described in Sambrook, J, Fritsch, E. F. and Maniatis, T. Molecular Cloning, a Laboratory Manual, Second edition, Cold Spring Harbor Laboratory Press (1989) and are well known in the art.

"Expression cassettes" and "expression vectors" are defined herein as DNA sequences that are required for the transcription of cloned DNA and the translation of their mRNAs in an appropriate host. Such vectors can be used to express prokaryotic and eukaryotic DNA in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

To employ the instant expression vectors the complete open reading frame of the gene of interest is cloned into the *NdeI* cloning site and the resulting plasmid is transformed into the target *Streptomyces* strain in which expression is to be studied. Integrants are selected by resistance to hygromycin. The presence of integrated vector and cloned gene may be verified by Southern analysis of the DNA prepared from hygromycin resistant strains. The resultant recombinant strains are very stable and addition of drug marker is not necessary to ensure

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maintenance of the construct during fermentation. Furthermore, the expression cassette along with the cloned gene may be placed on a low or high copy *Streptomyces* plasmid and transferred to any strain of interest for overexpression purposes required for biochemical and structural studies. A specific application of the vector is to express the gene encoding 31-O-methyltransferase, involved in methylation at position 31 of the immunosuppressive drug FK506, in *S. lividans*. The recombinant *S. lividans*, with integrated methyltransferase gene, successfully converted exogenously added 31-O-demethyl-FK506 to FK506, thus demonstrating functional expression of the cloned gene.

In a specific embodiment of the present invention, two integrative expression vectors pHM-6a and pHM-6b were designed and constructed. The two constructs in which the gene encoding 31-O-desmethylFK-506 O:methyltransferase (FKMT2), is under expression of a strong promoter, were incorporated into the genome of TK21 a strain of *Streptomyces lividans*. The resulting strains were then examined for the specific methylation of 31-O-desmethylFK-506 substrate which was added to the culture during fermentation. The purified fraction isolated from the culture was identified as FK-506 confirming expression of FKMT2 gene in a heterologous host. The developed strains thus prepared were fully capable of specific methylation of 31-O-desmethylFK-506.

Construction of the integrative expression cassettes pHM6a and pHM6b carrying the FKMT2 gene

The steps involved in the construction of integration expression vectors pHM6a and pHM6b, as schematically outlined in Figure 4, are described below.

- (1) A three kb SmaI fragment comprising the FKMT2 gene and flanking DNAs (sites 2 through 9 of Figure 1) was cloned into the SmaI site of pGEM-7Zf(-) (Promega, Madison, WI, USA) linearized with SmaI, resulting in plasmid pHM1.

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(2) A 1.8 kb BamHI-PstI fragment containing hygromycin resistance gene (hyg) was isolated from plasmid pIJ963 (Hopwood, D.A., et al. Genetic Manipulation of Streptomyces, A Laboratory Manual (1985)) and after filling the ends with T4-DNA polymerase,  
 5 was inserted into the polIK- filled EcoRI site of pHM1. This gave rise to plasmid pHM2 in which hyg gene was downstream from FKMT2 in the direction facing the methyltransferase (see pHM2 map in Figure 4).

(3) Three oligodeoxynucleotide primers PC1, PC2, and PC3  
 10 were synthesized on Applied Biosystems (Foster City, Calif., U.S.A. ) DNA synthesizer model 380A. The three oligonucleotides were then used in two PCR reactions to make RBS-a and RBS-b as follows:

(3a) Preparation of RBS-a

15 PC1 sense primer was a 59 mer of the sequence (SEQ ID NO:4)

5'-GG AAG CTT ACA GAA CCA CTC CAC AGG AGG  
ACC CAT ATG AGC GCC GCG GTG GAG ACG TTG

20 in which the underlined portion is derived from ribosome binding site region (-27 to -4) of *Streptomyces ramocissimus* elongation factor Tu (tuf1). Immediately upstream and adjacent to RBS sequence a HindIII recognition site (SEQ ID NO:5)

5'-AAGCTT

25 depicted in bold letters and on the 3' end a NdeI recognition site (SEQ ID NO:3)

5'-CATATG

30 depicted in bold letters were incorporated. The remaining nucleotide sequence of the primer corresponds to the nucleotide +4 to +24 at the N-terminal of FKMT2 coding region (depicted herein). The incorporation of NdeI at the translational start site changed the start codon of FKMT2 from GTG to ATG. The antisense primer PC2 was a 48 mer derived from the FKMT2 coding sequence corresponding to the complementary sequence to nucleotide 188 (5') to nucleotide 140 (3') (depicted herein) with the sequence (SEQ ID NO:6)

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ACG CTC GAG ATG AGC GAA GAG CGT GAA CAT  
GCC GAT GTT CGC GCC GAC

After amplification using PC1 and PC2 as primers and pHM1 as template DNA, the PCR products (220 bp) were purified and  
5 digested with HindIII and BglII and a 120 bp fragment was isolated and cloned between HindIII and BglII sites of pHM2 generating pHM3a.

(3b) Preparation of RBS-b

PC3 sense primer was a 62 mer of the sequence (SEQ ID  
10 NO:7)

5'-GGG AA GCT TAA GCT AAC GTA AGG AGG  
AAA AA CTA ATG AGCG CCG CGG TGG AGA CGT TGC GGC  
in which the underlined portion was according to a consensus RBS  
sequence characteristic of *E. coli* sequence (Gold, L. and Stormo, G.D.,  
15 Methods in Enzymology, 185, 89-93 (1990)). Like RBS-a, RBS-b has HindIII recognition site on the 5' end and an NdeI site on the 3' end.  
The remaining nucleotides, 3' to NdeI, is derived from the FKMT2 coding region nucleotide +4 to +28 (depicted herein).

Primer PC3 was used with primer PC2 in a PCR reaction  
20 with pHM1 as template and a fragment of 120 bp was isolated from HindIII-BglII digestion of the resulting PCR product, which was then inserted between similar sites of pHM2 producing pHM3b.

(4) Plasmid pIJ4070 which carries *ermE\** promoter  
25 ("PerME\*") (Bibb, M.J., et al., Fifth Int'l Symp. Genetics Ind. Microorganisms, 309-318 (1986)) was digested with NdeI and its 5'-overhangs were filled-in with polIK and recircularized to give pHM4 in which the NdeI site had been eliminated.

30 (5) The 4 kb XbaI-HindIII fragments of pHM3a and pHM3b were excised and the ends were filled with polIK and ligated between BamHI and HindIII sites of pHM4 after the ends were made blunt with polIK. This generated plasmids pHM5a and pHM5b in which the FKMT2

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gene was placed under transcriptional/translational control of *PermeE\** and RBS, respectively.

- 5 (6) Plasmids pHM5a and pHM5b were made integrative by inserting the 2.6 kb blunt-ended HindIII-cut mini-circle fragment (Lidiate, D.J., et al., Mol. and Gen. Genet., 203, 79-88 (1986)) into ScaI site producing pHM6a and pHM6b.

10 Construction of Expression Cassettes pHM7a and PHM7b and Integrative Expression Vectors pHM8a and PHM8b

- Expression cassettes pHM7a and pHM7b (of Figure 5) were constructed by inserting a synthetic polylinker NdeI-NcoI-XbaI-HindIII-BamHI between NdeI and BamHI sites of pHM5a and pHM5b (Figure5). This treatment introduced 3 additional unique sites into pHM7a and 7b, next to the NdeI recognition site. The synthetic polylinker was made as follow. Two complementary oligonucleotides PL1 (SEQ ID NO:8):

5'-TAT GCC ATG GCT CTA GAG AAG CTT G

and PL2 (SEQ ID NO:9):

5'-GAT CCA AGC TTC TCT AGA GCC ATG GCA

- 20 were designed, synthesized and annealed. The duplex oligo which had an NdeI site overhang on the 5' end and a BamHI site overhang on the 3' end was ligated between NdeI and BamHI sites of pHM5a and pHM5b which had been prepared as noted above.

- 25 Plasmids pHM5a and pHM5b (Figure 5) were cleaved with BamHI and NdeI and the larger (6.3 kb) fragment was isolated. This treatment removed most of the coding region of the FKMT2 and left behind its terminator region (Ter) which is within a 1.3 kb BamHI-SmaI fragment. The FKMT2 terminator contains several inverted repeats (two of which are marked in the FKMT2 sequence by underlined and overlined arrows) with the potential of forming stem and loop structures characteristics of rho-independent transcription termination regions of prokaryotic genes.

- 30 The polylinker, containing restriction recognition sites for NdeI, NcoI, XbaI, HindIII and BamHI, was then ligated between NdeI

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and BamHI sites of the isolated fragments to generate pHM7a and pHM7b.

Expression vectors pHM7a and 7b are multicopy *E. coli* plasmids (pUC18 derivatives) that carry 4 kb *Streptomyces* expression cassettes (Figure 5). The cassettes are comprised of PermE\*, RBS, polylinker, terminator and hyg resistance gene functional both in *E. coli* and *Streptomyces* as selectable marker. Furthermore, as shown in Figure 5, expression cassettes can be released as a 4 kb BglII fragment (marked by astricks) from pHM7a and pHM7b. Other usable sites are SstI on the 5' end and XhoI and PstI on the 3' end of the 4 kb expression cassette which can be utilized to liberate the expression cassette as well. The expression cassettes may be placed onto both low and high copy *Streptomyces* replicative plasmids for the overexpression of the genes of interest or they may be integrated into the genome via an integration element for introduction of single copy gene into the genome. One such an element is *Streptomyces coelicolor* A3 (Bibb, M.J., et al., Fifth Int'l Symp. Genetics of Ind. Microorganisms, 309-318 (1986)) mini-circle which is a 2.6 kb transposable element (Lidiate, D.J., et al., Mol. and Gen. Genet., 203, 79-88 (1986)) and carries an integration function which recognizes a specific site in the genome of several *Streptomyces* species (Lidiate, D.J., et al., Genetics of Industrial Microorganisms, 49-56 (1987)). The mini-circle sequence, when cloned into a vector, can direct integration of the entire construct into the chromosome through homologous recombination between chromosomal attachment (att) site and mini-circle att site resulting in the formation of a stable recombinant strain. The mini-circle has a wide host range ((Lidiate, D.J., et al., Genetics of Industrial Microorganisms, 49-56 (1987)) and several *Streptomyces* species such as *S. avermitilis*, *S. lividans*, *S. glaucescense*, *S. parvulus*, and many others referenced in Lidiate, D.J., et al., Mol. and Gen. Genet., 203, 79-88 (1986) have the att site for this element.

Furthermore, in cases in which *Streptomyces* species do not contain mini-circle att sites, another integration element may be used. This can be achieved by choosing a non-essential gene as a platform for

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the integration of the construct into the genome which requires cloning of the designated gene in place of the mini-circle in vectors pHM7a and pHM7b, followed by integration into the chromosome through homologous recombination between the vector copy and the  
5 chromosomal copy of the target gene. Any gene encoding a secondary metabolite enzyme may be employed for this purpose.

Integrative expression vectors pHM8a and pHM8b (Figure 5) were made by inserting a 2.6 kb *polIK*-treated HindIII-cut mini-circle fragment (Lidiate, D.J., et al., Genetics of Industrial  
10 Microorganisms, 49-56 (1987)) into ScaI site of pHM7a and pHM7b, as depicted in Figure 5.

Construction of recombinant strains *S. lividans*-pHM6a and  
*S. lividans*-pHM6b: Integration of pHM6a and pHM6b into the  
15 chromosome of *S. lividans*

Protoplasts of *Streptomyces lividans* TK21 (ATCC No. 55251) (Hopwood, D.A., et al., Genetic Manipulation of *Streptomyces*. A Laboratory Manual (1985)) were prepared and transformed with pHM6a and pHM6b DNA in two separate reactions. After overnight  
20 incubation at 30°C., plates were overlaid with nutrient soft agar containing hygromycin B sufficient to give a final concentration of 200 µg/ml (Hopwood, D.A., et al. Genetic Manipulation of *Streptomyces*. A Laboratory Manual (1985)). Plates were incubated for 2-3 additional days and hyg-resistant transformants were tested for functional  
25 expression of FKMT2 as follow.

In the general procedure for employing the present vectors, a two step cloning procedure is employed. In the first step the gene of interest is inserted into the polylinker 3' of the NdeI site. In the second step a small synthetic DNA encoding a small portion of the N-terminal  
30 region of the protein is prepared in which NdeI is incorporated at the translational start site. The size of the synthetic DNA depends on the presence of a sensible restriction site closest to the ATG site at the beginning of the gene. The synthetic fragment so prepared replaces the corresponding portion of the gene already cloned into the vector.



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Incorporation of NdeI site at the start site of translation permits expression of the proteins in their authentic nonfused form which ensures functionality of the expressed proteins. The constructed vector is inserted into *Streptomyces* species of choice via mini-circle or by  
5 other means described above.

Integrants may be selected by resistance to hygromycin. The presence of vector and cloned gene may then be verified by Southern analysis (Sambrook, J. Fritsch, E. F. and Maniatis, T. Molecular Cloning, a Laboratory Manual, Second edition, Cold Spring  
10 Harbor Laboratory Press (1989)) may be performed on the genomic DNA prepared from integrants. Integrated vectors are very stable and addition of drug is not necessary to keep the construct intact during fermentation. The presence of antibiotic drugmarker in the  
15 fermentation broth often causes problems by affecting the level of expression of genes of interest. Furthermore, in cases where the drug does not have any effect on the expression, the cost of drug can be a factor. Consequently, the use of the present integrative vector eliminates the deleterious effects on production and cost due to the  
20 presence of antibiotic. Moreover the constructed cassette can be placed on a *Streptomyces* replicating plasmid and transformed into the desired host strain for overexpression studies.

Expression of 31-O-desmethylFK-506 O:methyltransferase (FKMT2)

Employing pHM6a and pHM6b the gene (FKMT2)  
25 encoding 31-desmethyl FK506 methyltransferase was used to examine the potential of these vectors in expression of a foreign gene in *Streptomyces lividans*. As a result, two strains of *S. lividans*, SI/pHM6a and SI/pHM6b were obtained which were capable of conversion of the  
30 31-desmethyl FK506 to FK506 after addition of substrate to the culture broth during fermentation. HPLC, NMR and MASS analysis of the purified fraction from the culture extracts established the identity of the product as FK506 confirming functionality of the expressed methyltransferase.

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The nucleotide sequence of the FKMT2 coding sequence and the terminator region (1150 base pairs) are depicted below. The GTG translation start site at position +1 and the TAG translation stop codon at position 781 are depicted in bold letters. Two sets of inverted repeats at the 3'-end of the TAG codon are underlined and overlined by arrows, respectively. The nucleotide sequence of the FKMT2 sequence and the terminator region (SEQ ID NO:10) is

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1  CGATGAACGA GGGCCTCGGG CTTTTCAGCC CGGCCGAGCT GCGGGTCACC
10  51 TGGGGTGCCG T
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      GTGAGCGCC GCGGTGGAGA CGTTGCGGCT GCCGAACGGG
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      +89
101 ACGAAGGTCG CGCACATCAA CGCGGGCGAG GCGCAGTTCC TGTATCGGGA
      +139
151 GATCTTCACC GAGCGCTGCT ATCTGCGCCA CGGCGTGGAA CTGCGCCCCG
      +189
15  201 GTGACGTGGT GTTCGACGTC GGCGCGAACA TCGGCATGTT CACGCTCTTC
      +239
      +289
251 GCTCATCTCG AGCGTCCCGG CGTGACCGTG CACGCGTTTC AGCCCGCGCC
      +339
      +389
301 GGTGCCGTTC GCCGCGCTGC GGGCGAACGC GGCACAGTAC GGCATCTCGG
      +439
351 GCCGGGTGGA CCAGTGCGCG GTCTCCGACG AGCCCGGCGT ACGCAAGATG
      +489
20  401 ACGTTCTACC CCGACGCCAC GCTGATGTCC GGCTTCCATC CGGACGCCGC
      +539
      +589
451 GGCCCGCAAG GAGCTGTTGC GCACACTAGG CCTCAACGGC GGATACACCG
      +639
501 CCGAGGACGT CGACGGCATG CTCGCCAAC TGCCCGACTC GGGCGAGGAG
      +689
551 ATCGAGACCG CCGTGGTCCG CCTCTCCGAC ATCATCGCCG AGCGCCGCAT
      +739
25  601 CGCGACGATC GGCCTCCTGA AGGTGACGT GGAAAGGAGT GAACGGCAGG
      +783
      +839
651 TCCTCGCCGG CATCGAAGAA GCCGACTGGC CCCGCATCCG CCAGGTCGTC
      +889
701 GCGGAGGTCC ACGACGTGA GGGCGCGCTC GACGAGGTTC TCGCGCTGCT
      +939
751 GCGCGGCCAT GGCTTACCG TCGTCGCGA GCAGGATCCG CTGTTGCGCC
      +989
30  801 GCACGGACAT CCACCAGGTC GCCGCACGGC GTGCGAGCCA CTGA
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951 GAACACGTTT GTGAGGTGCT GTTCCACCGT GCTGGCCGTG ACGAACAGCT  
 1001 GCTCGGCGAT CTCCCTGTTC GTACGCCCCA CCGCGGCCAG CGCGGCCACC  
 1051 CGCCGCTCCG ACTCCGTCAG TGACGCGATC CGCTGCCCCA GCGTCGCGTC  
 5 1101 CCAGGCCCCG CCGATGTCCG AGGGCTCCGC GCCGAGCCGC CGCAGGAGCG

In particular, the nucleotide sequence of the FKMT2 coding sequence from position +1 and +783 is the following sequence (SEQ ID NO:11):

10	+1	GTGAGCGCC GCGGTGGAGA CGTTGCGGCT GCCGAACGGG	+39
		ACGAAGGTCG CGCACATCAA CGCGGGCGAG GCGCAGTTCC TGTATCGGGA	+89
		GATCTTCACC GAGCGCTGCT ATCTGCGCCA CGGCGTGAA CTGCGCCCCG	+139
		GTGACGTGGT GTTCGACGTC GGC CGAACA TCGGCATGTT CACGCTCTTC	+189
15		GCTCATCTCG AGCGTCCCGG CGTGACCGTG CACGCGTTCG AGCCCGCGCC	+239
		GGTGCCGTTT GCCGCGCTGC GGGCGAACGC GGCACAGTAC GGCATCTCGG	+289
		GCCGGGTGGA CCAGTGCGCG GTCTCCGACG AGCCCGGCGT ACGCAAGATG	+339
		ACGTTCTACC CCGACGCCAC GCTGATGTCC GGCTTCCATC CGGACGCCGC	+389
20		GGCCCGCAAG GAGCTGTTGC GCACACTAGG CCTCAACGGC GGATACACCG	+439
		CCGAGGACGT CGACGGCATG CTCGCCAAC TGCCCGACTC GGGCGAGGAG	+489
		ATCGAGACCG CCGTGGTCCG CCTCTCCGAC ATCATCGCCG AGCGCCGCAT	+539
		CGCGACGATC GGCTTCTCTGA AGGTGACGT GGAAAGGAGT GAACGGCAGG	+589
25		TCCTCGCCGG CATCGAAGAA GCCGACTGGC CCCGCATCCG CCAGGTCGTC	+639
		GCGGAGGTCC ACGACGTCGA GGGCGCGCTC GACGAGGTCG TCGCGCTGCT	+689
		GCGCGGCCAT GGCTTCACCG TCGTCGCCGA GCAGGATCCG CTGTTCCGCG	+739
		GCACGGACAT CCACCAGGTC GCCGCACGGC GTGCGAGCCA CTGA	+783
30			

The entire coding sequence of FKMT2, the gene encoding 31-desmethyl FK506-O-methyltransferase from *Streptomyces* (MA6548, ATCC 53770), was cloned into NdeI site of integrative vector shown in Figure 1A. with the help of PCR. Two constructs were made (pHM6a and pHM6b in Figure 4) in which RBS was within two

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different sequence contexts. Both vectors were then integrated into the genome of *Streptomyces lividans*, a heterologous host. Expression of the methyltransferase enzyme was monitored by the conversion of 31-desmethyl FK-506 to FK-506 after addition of substrate to the culture  
5 broth during fermentation. In both cases 100% conversion of 31-desmethyl FK-506 to FK-506 was observed. However pHM6b construct seemed to be slightly slower in its bioconversion ability. HPLC, NMR and MASS analysis of the culture extracts prepared from the pHM6a and pHM6b constructs confirmed the product to be FK-506.

10 The following examples are given for the purpose of illustrating the present invention and shall not be construed as being limitations on the scope or spirit of the instant invention.

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EXAMPLE 1General Methodology:Strains

- 5 1. *E. coli* strain JM109  
2. *Streptomyces* strain *S. lividans* TK21

*E. coli*:

10 All the methods, media and buffers used to handle *E. coli*  
were according to Sambrook et al. (Sambrook, J. Fritsch, E. F. and  
Maniatis, T., Molecular Cloning, a Laboratory Manual, Second edition,  
Cold Spring Harbor Laboratory Press (1989)) unless otherwise indicated.  
*E. coli* strains containing plasmids were grown in LB broth in the  
15 presence of antibiotic marker carried on the plasmid. The level of  
drugs used was 100 ug/ml for ampicillin, and 50 µg/ml of hygromycin  
B both obtained from Sigma. Plasmid preparation was done on 50 ml  
cultures using QIAGEN® columns (QIAGEN Inc. 9259 Eton Ave.  
Chatsworth, CA 91311). Preparation of *E. coli* competent cells for  
20 transformation, DNA manipulation such as restriction digestion, filling  
end reactions, ligation, agarose gel electrophoresis and hybridization by  
Southern analysis were carried out using standard methods described by  
Sambrook et al. (Sambrook, J. Fritsch, E. F. and Maniatis, T.,  
Molecular Cloning, a Laboratory Manual, Second edition, Cold Spring  
25 Harbor Laboratory Press (1989). Sequencing was conducted on double  
stranded DNA template by dideoxy chain termination method using  
sequenase version 2.0 (United States Biochemicals) following the  
recommendations supplied therewith. DNA fragment isolation was  
carried out using QIAEX® gel extraction protocol (QIAGEN Inc.)

30 *Streptomyces*:

All of the procedures, media and buffers used to handle  
*Streptomyces* were according to Hopwood et al. (Hopwood, D.A., et al.,  
Genetic Manipulation of *Streptomyces*, A Laboratory Manual (1985))

- 20 -

or standard modifications thereof. *Streptomyces* strains were grown in R5 broth for 2-3 days.

For plasmid preparation, strains were grown in R5 in the presence of drug as follows: thiostrepton at 10 µg/ml; and hygromycin B at 200 µg/ml. For protoplast preparations, glycine was included in R5 at 0.5%. Methods used for chromosomal DNA preparation, plasmid DNA isolation, protoplast preparation and transformation are described below. Other DNA manipulations were carried out as described as referenced herein for *E. coli* (Sambrook, J. Fritsch, E. F. and Maniatis, T., Molecular Cloning, a Laboratory Manual, Second edition, Cold Spring Harbor Laboratory Press (1989) and for *Streptomyces* (Hopwood, D.A., et al. Genetic Manipulation of *Streptomyces*. A Laboratory Manual (1985)).

15

## EXAMPLE 2

### *Streptomyces* Chromosomal DNA Isolation

*Streptomyces* cultures (50 ml), grown in R5 broth (Hopwood et al., Genetic Manipulation of *Streptomyces*. A Laboratory Manual (1985)), was centrifuged for 10 min and the supernatant was discarded. 20 ml of 10.3% sucrose was added, vortexed and then centrifuged for 10 min. The supernatant was discarded. Then 20 ml of lysozyme buffer (10.3% sucrose, 25 mM Tris-HCl pH 8.0, 25 mM EDTA and 5 mg/ml lysozyme) was added. The mixture was vortexed and incubated at 37°C for 45 min. Proteinase K was added to 50 µg/ml and incubated at 37°C for 15 min. Then, 2 ml of 20% SDS was added, mixed well and heated at 65°C for 10 min. After cooled to room temperature in ice, the sample was extracted with an equal volume of acid phenol:chloroform and centrifuged at 13,000 g for 20 min. The supernatant was transferred to a fresh tube with a 25 ml wide-tip pipet. The acid phenol:chloroform extraction was repeated one more time. A 1/10 volume of 3M NaOAc and 2 volumes of isopropanol were added, mixed and left at room temperature for 15 min. The mixture was centrifuged at 8,000 g for 10 min. The supernatant was discarded and

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the tube was dried with a kimwipe. 5 ml of TE (10 mM Tris-HCl pH 8.0 /1mM EDTA) was added to the pellet. After it was dissolved, RNAase A was added to 50 µg/ml and incubated at 37°C for 30 min. The mixture was extracted with 5 ml of neutral phenol:chloroform and centrifuged at 12,000g for 15 min. The supernatant was transferred to a fresh tube and same amount of chloroform was added. The mixture was vortexed and centrifuged at 8,000 g for 5 min. The supernatant was transferred to a new tube. A 1/10 volume of unbuffered 3 M NaOAc and equal volume of isopropanol were added and mixed. After leaving at room temperature for 15 min, the mixture was centrifuged at 8,000 g for 10 min. The supernatant was discarded. The pellet was washed with 10 ml of 70% ethanol (but care was taken not to vortex) and centrifuged at 8,000 g for 10 min. The supernatant was discarded. The inside of tube was dried with kimwipes and then dried in vacuum. The pellet was dissolved in 3 ml of TE and was stored at 4°C.

### EXAMPLE 3

#### Plasmid Preparation From *Streptomyces*

*Streptomyces* culture (50 ml) was centrifuged at 10,000 g for 15 min. The pellet was resuspended in 40 ml of 10.3% sucrose and centrifuged. The supernatant was discarded. Lysis buffer (20 ml) (10.3% sucrose, 25 mM Tris-HCl pH 8.0, 25 mM EDTA) containing 2 mg/ml lysozyme was added, vortexed and incubated at 37°C in water bath shaker for 45 min. Alkaline SDS (12 ml) (0.2 M NaOH/2% SDS) was added and mixed. The sample was incubated for 15 min at 70°C for small plasmids (under 12 kb) and 30 min at 55°C for larger ones (above 12kb) and then cooled to room temperature. Acid phenol:chloroform (equal volume) was added and vortexed for a few min. The mixture was centrifuged at 12,000 g for 15 min. The supernatant was transferred to a clean centrifuge tube. A 1/10 volume of 3M unbuffered NaOAc and equal volume of isopropanol were added, mixed and left at room temperature for 10 min. The mixture was centrifuged at 10,000 g for 15 min. The supernatant was poured off and the tube was wiped off.

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with kimwipes. The pellet was dissolved in 10 ml of 30 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA pH 8.0, then extracted with 5 ml of acid phenol:chloroform and spun at 12,000g for 15 min. The top layer was transferred to a new tube, extracted with 5 ml of chloroform and centrifuged again. The supernatant was transferred to a new tube. RNase A was added to 50 µg/ml and incubated at 37°C for 30 min. The sample was extracted with phenol:chloroform and chloroform as before. After centrifugation, the top layer was transferred to a new tube and the plasmid was precipitated with NaOAc/isopropanol as before. The plasmid DNA pellet was dissolved in 9 ml TE, 1 ml of 4 mg/ml EtBr. Then 10.3 g CsCl was added and dissolved completely. The mixture was transferred to a quick-seal tube. The tube was sealed and centrifuged at 37,000 rpm in Beckman 70.1 Ti rotor for 60 hours. The plasmid band was collected. The EtBr was extracted from the sample with the isopropanol saturated with TE and NaCl. The sample was dialyzed in TE overnight at 4°C. A 1/10 volume of 3M NaOAc pH 5.2 and 2.2 volume of 100% ethanol was added to the sample, mixed and placed at -20°C for at least 1 hour, followed by centrifugation at 10,000 g for 20 min. The pellet was washed once with 70% ethanol and then dried in vacuum. The plasmid DNA was resuspended in 100-500 µl of TE and stored at -20°C.

#### EXAMPLE 4

##### 25 Protoplast preparation:

*Streptomyces* cultures (50 ml) grown in R5 in the presence of glycine (0.5%) were harvested for 10 min in tabletop centrifuge. The cells were washed with 10.3% sucrose and centrifuged for 10 min as above. The pellet was suspended in 10 ml "P" medium (Hopwood et al., Genetic Manipulation of *Streptomyces*, A Laboratory Manual (1985)) containing 1.5 mg/ml lysozyme (Sigma) and incubated in a 30°C water bath shaker for 30-75 min. After 15 min incubation, and every 10 min thereafter, cell suspension was checked under microscope to determine completion of protoplast formation. Protoplasts were then



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filtered through a spore filter tube and spun for 7 min, at 3,000 rpm. The supernatant was poured off and pellet was resuspended in 10 ml of "P" buffer followed by 7 min centrifugation as above. The pellet was then resuspended gently in 3-5 ml of "P" buffer and stored in small  
5 aliquats at 80°C.

### EXAMPLE 5

#### Transformation:

10 Plasmid DNA or ligation mixes (100-500 ng) was added to 50 ul of protoplasts. 500 ul transformatoin buffer (25% PEG 1000 in "P" buffer (Hopwood, D.A., et al., Genetic Manipulation of  
*Streptomyces*, A Laboratory Manual (1985))) was added to the  
15 protoplast/DNA mixture and mixed gently. The transformation mixture (100 ul) was then plated on R2YE plates and incubated at 30°C overnight. Plates were then overlaid (3ml) with soft nutrient agar (Hopwood et al., Genetic Manipulation of *Streptomyces*, A Laboratory Manual (1985)) in the presence of antibiotic marker carried on the  
20 plasmid and incubated for an additional 3-4 days.

### EXAMPLE 6

#### Bioconversion of 31-O-desmethylFK-506 to FK-506

25 The gene (FKMT2) encoding 31-O-desmethylFK-506 O:methyltransferase was introduced into TK21, a strain of *Streptomyces lividans*, as described above. The resulting transformant was isolated and was grown in the seed and bioconversion media. One hundred milligrams of 31-O-desmethylFK-506 was then dissolved in 50 µl DMSO and the solution was added to the 500 ml bioconversion culture  
30 at the time of the transfer of the seed culture. At different time intervals, an aliquot of the bioconversion culture was withdrawn and examined for the formation of FK-506. The methylation of the 31-O-desmethylFK-506 started at about 18th hour of the incubation and continued until 65th hour, when there was complete conversion of the

- 24 -

substrate. The conversion of the 31-O-desmethylFK-506 into FK-506 was also measured using established HPLC procedures. The conversion was basically quantitative with no sign of side product formation. In order to confirm the nature of the bioconversion product, 500 ml  
5 bioconversion culture was harvested after 66 hours. The culture was mixed with an equal volume of methanol and centrifuged. The resulting supernatant was extracted with methylene chloride and the organic phase was recovered. This extract was concentrated to dryness under reduced pressure and the residue was suspended in 25 ml of 25%  
10 acetonitrile. The resulting solution was applied on a semi-preparative reverse-phase column and the column was developed with a gradient of acetonitrile in water. The desired product was eluted with 60-80% acetonitrile which was worked-up to yield 65 mg of the purified FK-506 (NMR and mass spectra obtained for the isolated product were  
15 identical to those of the standard, FK-506).

While the invention has been described and illustrated with reference to certain particular embodiments thereof, those skilled in the art will appreciate that various adaptations, changes, modifications,  
20 substitutions, deletions, or additions of procedures and protocols may be made without departing from the spirit and scope of the invention. It is intended, therefore, that the invention be defined by the scope of the claims which follow and that such claims be interpreted as broadly as is reasonable.

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- 25 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: MOTAMEDI, HAIDEH  
SHAFIEE, ALI
- (ii) TITLE OF INVENTION: EXPRESSION CASSETTES USEFUL IN  
CONSTRUCTION OF INTEGRATIVE AND REPLICATIVE EXPRESSION  
VECTORS FOR STREPTOMYCES
- (iii) NUMBER OF SEQUENCES: 11
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: J. ERIC THIES
  - (B) STREET: P.O. BOX 2000, 126 E. LINCOLN AVENUE
  - (C) CITY: RAHWAY
  - (D) STATE: NJ
  - (E) COUNTRY: USA
  - (F) ZIP: 07065
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: THIES, J. ERIC
  - (B) REGISTRATION NUMBER: 35,382
  - (C) REFERENCE/DOCKET NUMBER: 19132
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (908) 594-3904
  - (B) TELEFAX: (908) 594-4720

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 7 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGGAGGA 7

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGGAGGA 7

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATATG 6

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGAAGCTTAC AGAACCCTC CACAGGAGGA CCCATATGAG CGCCGCGGTG GAGACGTTG 59

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAGCTT 6

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACGCTCGAGA TGAGCGAAGA GCGTGAACAT GCCGATGTTT GCGCCGAC 48

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid

- 28 -

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGAAGCTTA AGCTAACGTA AGGAGGAAAA ACTAATGAGC GCCGCGGTGG AGACGTTGCG 60  
GC 62

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TATGCCATGG CTCTAGAGAA GCTTG 25

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GATCCAAGCT TCTCTAGAGC CATGGCA 27

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## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1150 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGATGAACGA GGGCCTCGGG CTTTTCAGCC CGGCCGAGCT GCGGGTCACC TGGGGTGCCG	60
TGTGAGCGCC GCGGTGGAGA CGTTGCGGCT GCCGAACGGG ACGAAGGTCG CGCACATCAA	120
CGCGGGCGAG GCGCAGTTCC TGTATCGGGA GATCTTCACC GAGCGCTGCT ATCTGCGCCA	180
CGCGGTGGAA CTGCGCCCCG GTGACGTGGT GTTCGACGTC GGC CGGAACA TCGGCATGTT	240
CACGCTCTTC GCTCATCTCG AGCGTCCCG CGTGACCGTG CACGCGTTCG AGCCCGCGCC	300
GGTGCCGTTC GCCCGCTGC GGGCGAACGC GGCACAGTAC GGCATCTCGG GCCGGGTGGA	360
CCAGTGCGCG GTCTCCGACG AGCCCGGCGT ACGCAAGATG ACGTTCTACC CCGACGCCAC	420
GCTGATGTCC GGCTTCCATC CGGACGCCGC GGCCCGCAAG GAGCTGTTGC GCACACTAGG	480
CCTCAACGGC GGATACACCG CCGAGGACGT CGACGGCATG CTCGCCAAC TGCCCGACTC	540
GGCGGAGGAG ATCGAGACCG CCGTGGTCCG CCTCTCCGAC ATCATCGCCG AGCGCCGCAT	600
CGCGACGATC GGCTCCTGA AGGTCGACGT GGAAAGGAGT GAACGGCAGG TCCTCGCCGG	660
CATCGAAGAA GCCGACTGGC CCCGCATCCG CCAGGTCGTC GCGGAGGTCC ACGACGTCGA	720
GGGCGCGCTC GACGAGGTCG TCGCGCTGCT GCGCGGCCAT GGCTTCACCG TCGTCGCCGA	780
GCAGGATCCG CTGTTGCGCG GCACGGACAT CCACCAGGTC GCCGCACGGC GTGCGAGCCA	840
CTGAGCCGCC GGGGCGCGGC TACCCGCACC GGCGGTGCGG GTCCGCGGGC TCGCCGACGT	900
CGGCCAGTTC CTTGGAAGC TGCTGGCGGC CCTTCACCGC CAGCTTTGCG GAACACGTTC	960
GTGAGGTGCT GTTCCACCGT GCTGGCCGTG ACGAACAGCT GCTCGGCGAT CTCCCTGTTC	1020
GTACGCCCCA CCGCGGCCAG CGCGGCCACC CGCCGCTCCG ACTCCGTCAG TGACGCGATC	1080
CGCTGCCCCA GCGTCGCGTC CCAGGCCCCG CCGATGTCCG AGGGCTCCGC GCCGAGCCGC	1140

- 30 -

CGCAGGAGCG

1150

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 783 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTGAGCGCCG CGGTGGAGAC GTTGGGGCTG CCGAACGGGA CGAAGGTCGC GCACATCAAC	60
GCGGGCGAGG CGCAGTTCCT GTATCGGGAG ATCTTCACCG AGCGCTGCTA TCTGCGCCAC	120
GGCGTGGAAC TGCGCCCCGG TGACGTGGTG TTCGACGTCG GCGCGAACAT CGGCATGTTC	180
ACGCTCTTCG CTCATCTCGA GCGTCCCGGC GTGACCGTGC ACGCGTTCGA GCCCCGCGCG	240
GTGCCGTTTCG CCGCGCTGCG GCGGAACGCG GCACAGTACG GCATCTCGGG CCGGCTGGAC	300
CAGTGCGCGG TCTCCGACGA GCCCCGCGTA CGCAAGATGA CGTTCCTACCC CGACGCCACG	360
CTGATGTCCG GCTTCCATCC GGACGCCGCG GCCCCGAAGG AGCTGTTGCG CACACTAGGC	420
CTCAACGGCG GATACACCGC CGAGGACGTC GACGGCATGC TCGCCCAACT GCCCGACTCG	480
GGCGAGGAGA TCGAGACCGC CGTGGTCCGC CTCTCCGACA TCATCGCCGA GCGCCGCATC	540
GCGACGATCG GCCTCCTGAA GGTGACGCTG GAAAGGAGTG AACGGCAGGT CCTCGCCGGC	600
ATCGAAGAAG CCGACTGGCC CCGCATCCGC CAGGTCGTGC CGGAGGTCCA CGACGTCGAG	660
GGCGCGCTCG ACGAGGTCGT CGCGCTGCTG CGCGGCCATG GCTTCACCGT CGTCGCCGAG	720
CAGGATCCGC TGTTCGCCGG CACGGACATC CACCAGGTCG CCGCACGGCG TCGAGCCAC	780
TGA	783

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WHAT IS CLAIMED IS:

1. An integrative expression cassette for the expression of a gene of interest in *Streptomyces* comprising:

- 5           (1) a strong constitutive promoter PermE\*;  
            (2) a synthetic ribosome binding site derived from the RBS region of the gene encoding elongation factor Tu (*tufI*) of *Streptomyces ramocissimus*;  
            (3) an ATG start codon contained within the *NdeI* cloning site;  
10          (4) a multiple cloning site, which gives additional cloning sites into which a gene of interest may be cloned;  
            (5) a transcription terminator derived from the terminator region of a methyltransferase gene involved in the biosynthesis of FK-506;  
15          in association with:  
            (i) a hygromycin resistance gene which acts as selectable marker by inactivating the drug hygromycin B;  
            (ii) a minicircle as an integration element; and  
20          (iii) a Col E1 replicon;

wherein the components (1) - (5) are associated in the specified order and each of the components (i), (ii), and (iii) may be independently associated either upstream or downstream of components (1) - (5).

25           2. The integrative expression cassette of Claim 1 wherein the minicircle is replaced by any non-essential gene and is used as an integration element.

30           3. The integrative expression cassette of Claim 1 wherein the strong constitutive promoter PermE\* is a promoter-up mutant derived from the wild type promoter for erythromycin resistance gene of *Saccharopolyspora erythrea*.

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4. The integrative expression cassette of Claim 1 wherein the minicircle is a *Streptomyces* integration element derived from *Streptomyces coelicolor*.

5. An integrative expression cassette for the expression of a gene of interest in *Streptomyces* comprising:

- (1) a strong constitutive promoter PermE\*;
- (2) a synthetic ribosome binding site derived from a consensus *E. coli*-RBS sequence;
- (3) an ATG start codon contained within the *NdeI* cloning site;
- (4) a multiple cloning site, which gives additional cloning sites into which a gene of interest may be cloned;
- (5) a transcription terminator derived from the terminator region of a methyltransferase gene involved in the biosynthesis of FK-506;

in association with:

- (i) a hygromycin resistance gene which acts as selectable marker by inactivating the drug hygromycin B;
- (ii) a minicircle as an integration element; and
- (iii) a Col E1 replicon;

wherein the components (1) - (5) are associated in the specified order and each of the components (i), (ii), and (iii) may be independently associated either upstream or downstream of components (1) - (5).

6. The integrative expression cassette of Claim 5 wherein the minicircle is replaced by any non-essential gene and is used as an integration element.

7. The integrative expression cassette of Claim 5 wherein the strong constitutive promoter PermE\* is a promoter-up mutant derived from the wild type promoter for erythromycin resistance gene of *Saccharopolyspora erythrea*.

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8. The integrative expression cassette of Claim 5 wherein the minicircle is a *Streptomyces* integration element derived from *Streptomyces coelicolor*.

- 5                   9. An expression cassette for the overexpression of a gene of interest in *Streptomyces* comprising:
- (1) a strong constitutive promoter, Perm E\*, derived from erythromycin resistance gene of *Saccharopolyspora erythraea*;
  - 10           (2) a synthetic ribosomal binding site (RBS) which include the heptamer (SEQ ID NO:2) AGGAGGA;
  - (3) a translation start site/cloning site which is a *NdeI* restriction site bearing a (SEQ ID NO:3) CATATG
  - 15                   recognition site and that serves both as cloning site and translation start site;
  - (4) a multiple cloning site; and
  - (5) a terminator derived from the terminator region of the
  - 20                   gene encoding FK506 methyltransferase, FKMT2 (isolated from MA6548) that is located downstream from the cloning site to prevent readthrough transcription;
  - in association with:
  - (i) a drug marker, which is hygromycin
  - 25                   phosphotransferase and which is expressed both in *E. coli* and *Streptomyces* and confers hygromycin resistance to the host by inactivation of hygromycin B;
  - wherein the components (1) - (5) are associated in the specified order
  - 30                   and the component (i) may be associated either upstream or downstream of components (1) - (5).

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10. The expression cassette of Claim 9 wherein the synthetic ribosomal binding site (RBS) is present in the sequence context as depicted in sequence context (a) of Figure 3.

5           11. The expression cassette of Claim 9 wherein the synthetic ribosomal binding site (RBS) is present in the sequence context as depicted in sequence context (b) of Figure 3.

10           12. A *Streptomyces* host cell containing the integrative expression cassette of Claim 1.

          13. A *Streptomyces* host cell containing the integrative expression cassette of Claim 5.

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14. A process for using the integrative expression cassette of Claim 1 comprising cloning the integrative expression cassette of Claim 1 into a *Streptomyces* chromosome.

5           15. The process of Claim 14 wherein the cloning is used in the development of a bioconverting strain.

16. The process of Claim 14 wherein the cloning is used in the production of a catalytic protein.  
10

17. A process for using the integrative expression cassette of Claim 5 comprising cloning the integrative expression cassette of Claim 5 into a *Streptomyces* chromosome.

15           18. The process of Claim 17 wherein the cloning is used in the development of a bioconverting strain.

19. The process of Claim 17 wherein the cloning is used in the production of a catalytic protein.  
20

20. A process for using the expression cassette of Claim 9 comprising cloning the expression cassette of Claim 9 into a *Streptomyces* replicative vector.

25           21. The process of Claim 20 wherein the cloning is used in the development of a bioconverting strain.

22. The process of Claim 20 wherein the cloning is used in the production of a catalytic protein.  
30

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23. An isolated DNA molecule encoding the enzyme  
31-O-desmethylFK-506 O:methyltransferase (FKMT2) and its  
terminator region comprising the nucleotide sequence (SEQ ID NO:10):

```

5      1 CGATGAACGA GGGCCTCGGG CTTTTCAGCC CGGCCGAGCT GCGGGTCACC
      +1
      51 TGGGGTGCCG TGTGAGCGCC GCGGTGGAGA CGTTGCGGCT GCCGAACGGG
      +89
     101 ACGAAGGTCG CGCACATCAA CGCGGGCGAG GCGCAGTTCC TGTATCGGGA
      +139
     151 GATCTTCACC GAGCGCTGCT ATCTGCGCCA CGGCGTGGAA CTGCGCCCCG
      +189
     201 GTGACGTGGT GTTCGACGTC GGCGCGAACA TCGGCATGTT CACGCTCTTC
      +239
    10  251 GCTCATCTCG AGCGTCCCCG CGTGACCGTG CACGCGTTCC AGCCCCGCGC
      +289
     301 GGTGCCGTTC GCCGCGCTGC GGGCGAACGC GGCACAGTAC GGCATCTCGG
      +339
     351 GCCGGGTGGA CCAGTGCGCG GTCTCCGACG AGCCCCGGCGT ACGCAAGATG
      +389
     401 ACGTTCTACC CCGACGCCAC GCTGATGTCC GGCTTCCATC CGGACGCCGC
      +439
    15  451 GGCCCGCAAG GAGCTGTTGC GCACACTAGG CCTCAACGGC GGATACACCG
      +489
     501 CCGAGGACGT CGACGGCATG CTCGCCAAC TGCCCCACTC GGGCGAGGAG
      +539
     551 ATCGAGACCG CCGTGGTCCG CCTCTCCGAC ATCATCGCCG AGCGCCGCAT
      +589
     601 CGCGACGATC GGCCTCCTGA AGGTCGACGT GGAAAGGAGT GAACGGCAGG
      +639
    20  651 TCCTCGCCCG CATCGAAGAA GCCGACTGGC CCCGCATCCG CCAGGTCGTC
      +689
     701 GCGGAGGTCC ACGACGTCGA GGGCGCGCTC GACGAGGTCC TCGCGCTGCT
      +739
     751 GCGCGGCCAT GGCTTCACCG TCGTCGCCGA GCAGGATCCG CTGTTCCGCC
      +783
     801 GCACGGACAT CCACCAGGTC GCCGCACGGC GTGCGAGCCA CTGAGCCGCC
    25  851 GGGGCGCGGC TACCCGCACC GGCGGTGCGG GTCCGCGGGC TCGCCGACGT
     901 CGGCCAGTTC CTTGGAAGC TGCTGGCGGC CCTTCACCGC CAGCTTTGCG
     951 GAACACGTTT GTGAGGTGCT GTTCCACCGT GCTGGCCGTG ACGAACAGCT
    1001 GCTCGGCGAT CTCCTGTTC GTACGCCCGA CCGCGGCCAG CGCGGCCACC
    30 1051 CGCCGCTCCG ACTCCGTCAG TGACGCGATC CGCTGCCCCA GCGTCGCGTC
    1101 CCAGGCCCCG CCGATGTCCG AGGGCTCCGC GCCGAGCCGC CGCAGGAGCG .

```

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24. An isolated DNA molecule encoding the enzyme  
31-O-desmethylFK-506 O:methyltransferase (FKMT2) comprising the  
nucleotide sequence (SEQ ID NO:11):

```
5          +1                                     +39
          GTGAGCGCC GCGGTGGAGA CGTTGCGGCT GCCGAACGGG
          +89
          ACGAAGGTCG CGCACATCAA CGCGGGCGAG GCGCAGTTCC TGTATCGGGA
          +139
          GATCTTCACC GAGCGCTGCT ATCTGCGCCA CGGCGTGGAA CTGCGCCCCG
          +189
10         GTGACGTGGT GTTCGACGTC GGCGCGAACA TCGGCATGTT CACGCTCTTC
          +239
          GCTCATCTCG AGCGTCCCGG CGTGACCGTG CACGCGTTTC AGCCCGCGCC
          +289
          GGTGCCGTTC GCCGCGCTGC GGGCGAACGC GGCACAGTAC GGCATCTCGG
          +339
          GCCGGGTGGA CCAGTGCGCG GTCTCCGACG AGCCCGGCGT ACGCAAGATG
          +389
15         ACGTTCTACC CCGACGCCAC GCTGATGTCC GGCTTCCATC CGGACGCCGC
          +439
          GGCCCGCAAG GAGCTGTTGC GCACACTAGG CCTCAACGGC GGATACACCG
          +489
          CCGAGGACGT CGACGGCATG CTCGCCAAC TGCCCGACTC GGGCGAGGAG
          +539
          ATCGAGACCG CCGTGGTCCG CCTCTCCGAC ATCATCGCCG AGCGCCGCAT
          +589
20         CGCGACGATC GGCCTCCTGA AGGTCGACGT GGAAAGGAGT GAACGGCAGG
          +639
          TCCTCGCCGG CATCGAAGAA GCCGACTGGC CCCGCATCCG CCAGGTCGTC
          +689
          GCGGAGGTCC ACGACGTCGA GGGCGCGCTC GACGAGGTTC TCGCGCTGCT
          +739
          GCGCGGCCAT GGCTTCACCG TCGTCGCCGA GCAGGATCCG CTGTTGCCCG
          +783
25         GCACGGACAT CCACCAGGTC GCCGCACGGC GTGCGAGCCA CTGA

30
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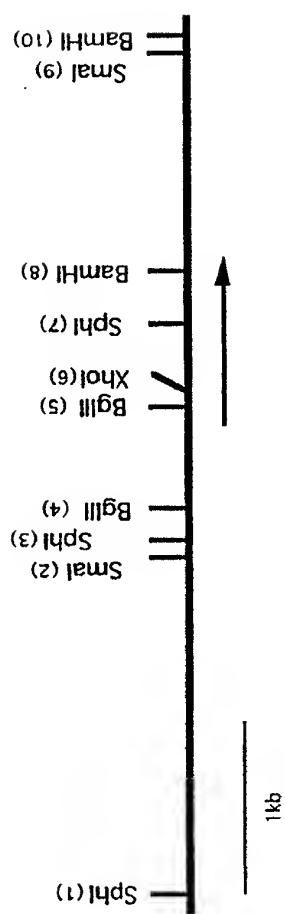


FIG.1

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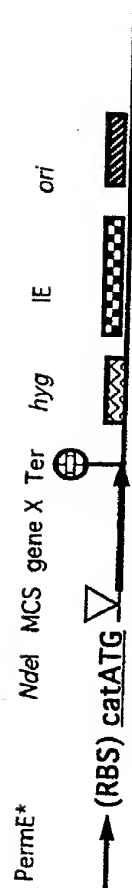


FIG. 2

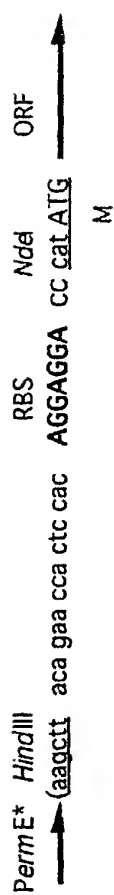


FIG.3 A

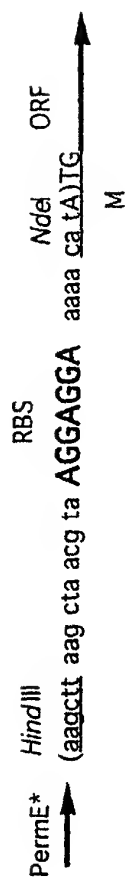


FIG.3B

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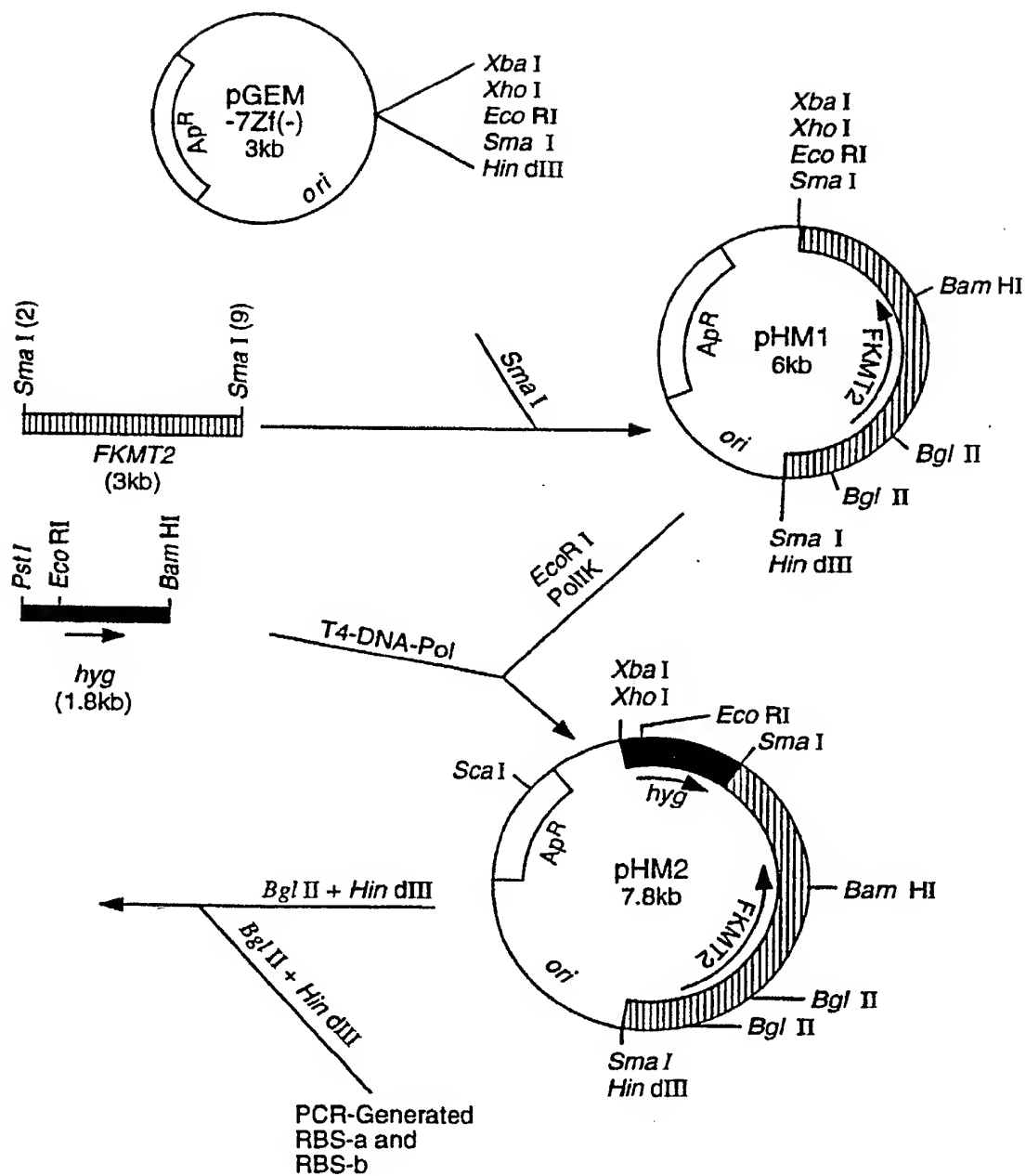


FIG.4A

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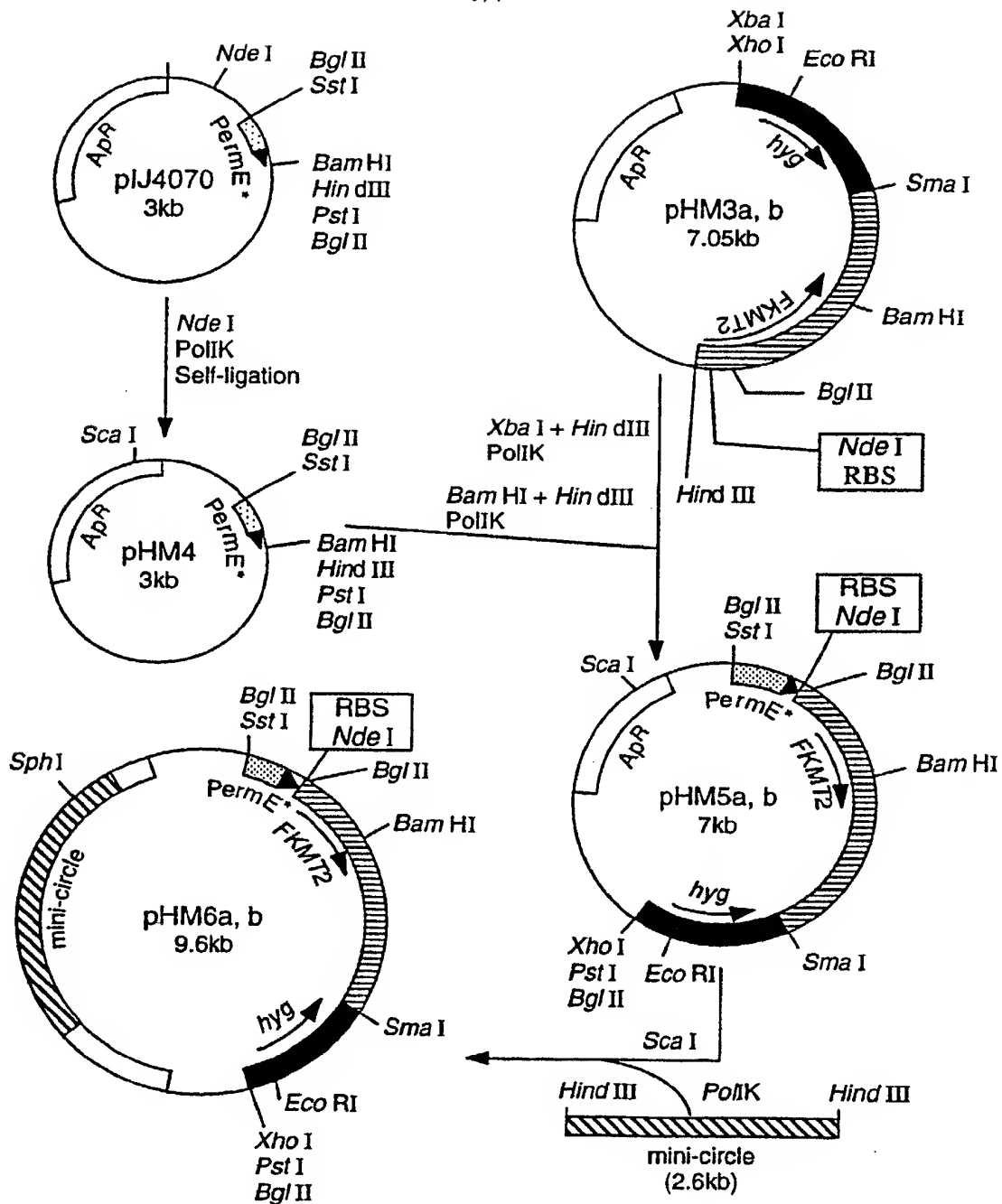


FIG.4B

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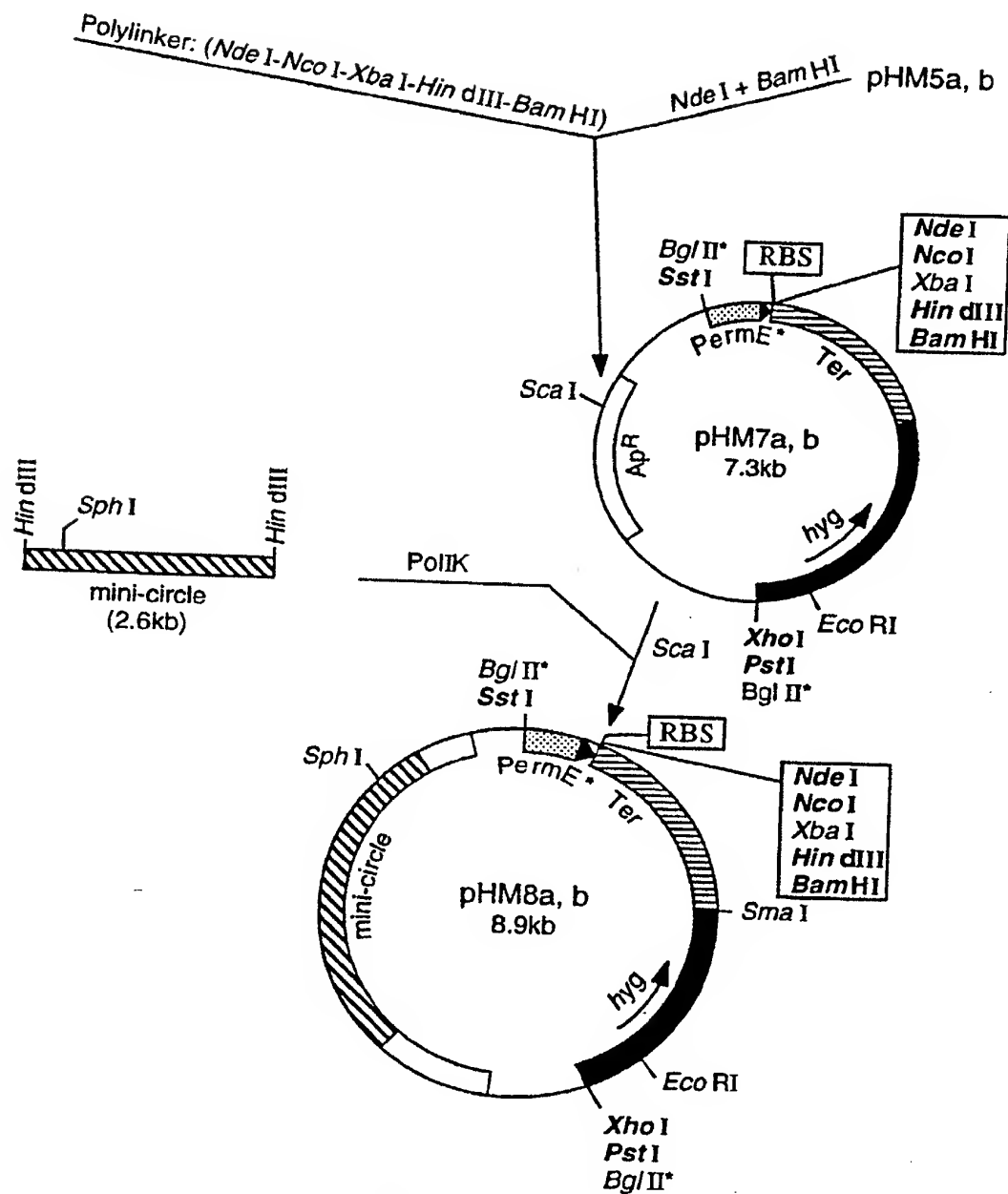
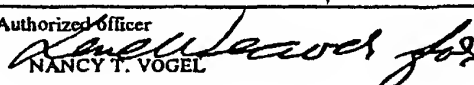


FIG. 5

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/07784

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(6) : C12N 1/21, 15/52, 15/63, 15/76 US CL : 435/320.1, 172.3, 252.35; 536/23.2 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/320.1, 172.3, 252.35; 536/23.2 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, DIALOG		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,264,355 (SHAFIEE ET AL) 23 November 1993, see entire document.	1-24
Y	Biochemical and Biophysical Research Communications, Volume 117, No. 1, issued 30 November 1993, Malpartida et al., "Molecular Cloning and Expression in Streptomyces Lividans of a Hygromycin B Phosphotransferase Gene from Streptomyces Hygroscopicus", pages 6-12, see entire article.	1-22
Y	US, A, 5,063,155 (COX ET AL) 05 November 1991, see entire document.	1-24
Y, P	US, A, 5,326,858 (LICHENSTEIN ET AL) 05 JULY 1994, column 9, line 41 through column 12, line 45.	1-24
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be part of particular relevance "E" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family		
Date of the actual completion of the international search 13 SEPTEMBER 1995		Date of mailing of the international search report 12 OCT 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231		Authorized Officer  NANCY T. VOGEL

# INTERNATIONAL SEARCH REPORT

Int. application No.  
PCT/US95/07784

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Gene, Volume 119, issued 1992, Inokoshi et al., "Cloning and Sequencing of the Aculeacin A acylase-encoding Gene From Actinoplanes utahensis and Expression in Streptomyces lividans", pages 29-35, see entire article.	1-22
Y	Molecular and General Genetics, Volume 203, issued 1986, Lydiate et al., "A 2.6 kb DNA Sequence of Streptomyces coelicolor A3(2) Which Functions as a Transposable Element", pages 79-88, see entire article.	1-22
Y	Microbiology, Volume 140, issued 1994, Vijgenboom et al., "Three tuf-like Genes in the Kirromycin Producer Streptomyces ramocissimus", pages 983-998, see entire article.	1-22
Y	POUWELS et al., "CLONING VECTORS, A LABORATORY MANUAL" published 1985 by Elsevier (Amsterdam), pages III-A-ii-1 and III-A-i-6, see pages III-A-ii-1 and III-A-i-6.	1-22
Y	Journal of Bacteriology, Volume 170, No. 5, issued May 1988, Omer et al., "Site-Specific Insertion of Biologically Functional Adventitious Genes into the Streptomyces lividans Chromosome", pages 2174-2184, see entire article.	1-22

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